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Differential Responses in Human Subcutaneous and Skeletal Muscle Vascular Beds to Critical Limb Ischaemia

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Objectives: *to investigate the effects of chronic ischaemia on the subcutaneous and the skeletal muscle resistance vasculature. To understand the redistribution of available blood in the ischaemic limb.*
Methods: *human subcutaneous and skeletal muscle resistance arteries were obtained from limbs amputated for critical limb ischaemia and studied under isobaric conditions using pressure myography. Morphological measurements of wall and lumen were analysed using light microscopy and image analysis. Vasoconstrictor responses to potassium and adrenoceptor agonists were used to measure functional status. Noradrenaline re-uptake mechanisms and α_1-selectivity were investigated.*
Results: *both human skeletal muscle and subcutaneous resistance arteries undergo a severe atrophy of the arterial wall in ischaemic conditions. However, whereas subcutaneous resistance arteries become less able to vasoconstrict to adrenoceptor stimulation, the response of skeletal muscle resistance arteries becomes exaggerated and significantly augmented. This is true in response to both the endogenous vasoconstrictor noradraline and the α_1-selective adrenoceptor agonist phenylephrine.*
Conclusions: *hypersensitivity to circulating catecholamines in the skeletal muscle vascular resistance bed may contribute to the progression of ischaemic disease by differentially diverting available blood to the subcutaneous tissue to the detriment of skeletal muscle perfusion.*

Key Words: Critical limb ischaemia; Resistance arteries; Pressure myography; Noradrenaline.

Introduction

In critical limb ischaemia (CLI) diffuse atherosclerotic plaques in the large communicating arteries create a significant resistance to blood flow.[1] Consequently, blood perfusion pressure is not high enough to overcome the resistance offered by successive vascular beds, and therefore leads to distal limb hypoperfusion and ischaemia.[1,2] The inadequate supply of available blood is then distributed between the tissues of the distal limb, presumably following the path of least resistance. How this path is determined remains to be elucidated.

An early study of peripheral arterioles in CLI subjects showed that they were relatively insensitive to vasodilator stimuli. This was generally taken to indicate that arterioles in CLI subjects may be maximally dilated.[3] As well as vasodilator paralysis, observations of capillary collapse (pallor) in raised ischaemic limbs and lower-limb suffusion (rubor) on standing have given the impression that arterioles of CLI subjects are also generally insensitive to vasoconstrictor stimuli.[4] With such general dysfunction these studies would suggest that resistance to flow is equally reduced throughout the limb. However, the sequelae of CLI, paraesthesia, tissue breakdown, gangrene and necrosis have all been identified as being the result of circulatory abnormalities within the local nutritive microcirculation of the subcutaneous and skin vascular beds.[5,6] Capillary microscopy, laser Doppler fluxmetry, fluorescein angiography/transcutaneous oxygen measurement (tcO_2), and more recently small vessel wire myography have all identified pathophysiological processes at the subcutaneous resistance or microcirculatory level.[7–10] At present, there are no data available which describe the effect of CLI on the other important vascular bed in the limb, the skeletal muscle vascular bed. Until this is available, it is difficult to understand how blood is distributed in the ischaemic limb.

We have therefore used pressure myography to study the effects of CLI on the structure and function

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of skeletal muscle resistance arteries and compared this with subcutaneous resistance levels. The overall aim of this study was to determine if both resistance beds were similarly affected by the ischaemic environment.

Materials and Methods

Resistance arteries were obtained from 43 patients (19 male, 24 female, mean age 69 ± 2 years; mean blood pressure $137 \pm 4/73 \pm 2$ mmHg) undergoing amputation (18 above knee and 25 below knee) for CLI. Of the 43 patients, 21 were of diabetic status (11 non-insulin-dependent diabetes mellitus (NIDDM); 10 IDDM). The study was approved by the appropriate Ethics Committee and each patient gave informed consent. The patients studied were all diagnosed as having CLI as defined in the European Consensus Document.[11] This defines CLI as persistently occurring rest pain and/or gangrene of the foot or toes plus an ankle systolic BP <50 mmHg and/or toe systolic BP <30 mmHg; transcutaneous oxygen pressure of the ischaemic area ($tcPO_2$) ≤ 10 mmHg.[11]

Biopsy procedure

Immediately following amputation, 1 cm^3 biopsies were isolated from four distinct anatomical sites: proximal and distal subcutaneous and proximal and distal skeletal muscle. The proximal biopsies were isolated ~ 2 cm below the level of amputation. The distal subcutaneous biopsies were isolated from the subcutaneous tissue directly inferior to the medial malleolus. Distal skeletal muscle biopsies were isolated from the distal portion of the soleus muscle. Arteries isolated from the proximal portion of the amputated leg have been classified as non-ischaemic and those isolated from the distal portion of the leg as ischaemic. This classification is based on the clinical determination of the amputation level, i.e. a level of normal blood perfusion at which complete and rapid healing of the stump can be reliably predicted. Each biopsy was immediately placed in ice-cold physiological saline solution (PSS) and transferred to the vascular laboratory. Resistance-size arteries were isolated from each biopsy and cleaned of any adherent tissue under a dissection microscope (Zeiss Stemi 2000).

Experimental technique and protocols

Figure 1 shows a representative resistance artery in the passive (Fig. 1A) and active (Fig. 1B) states *in situ* on the Danish MyoTech P100 pressure myograph system. The myograph block containing the double cannulated and pressurised arteries is placed on the stage of a Zeiss axiovert 25C inverted microscope. A real-time image of the artery (752×582 pixels) was captured by a high-resolution 16-bit CCD Sony XC-73CE monochrome video camera attached to the microscope's third ocular tube. A Data Translation's digital DT3157 Mach frame grabber processed the digital image. The final viewed magnification was, for pharmacology, $\times 100$ ($\times 5$ objective) and for morphology, $\times 200$ ($\times 10$ objective). In-line pressure transducers recorded left in-flow and right in-flow pressures. A force transducer with a sensitivity of 0.1 mN measured longitudinal tension between the microcannulae and any force deviations along the longitudinal axis of the artery. The video image was analysed using Vessel View software (Danish MyoTech), which used edge detection algorithms to measure external and internal diameters, and wall thickness. Time, mean pressure, longitudinal force, temperature, flow and manual interventions were also recorded. Data was acquired at intervals of 1 second and recorded on an IBM personal computer.

Isolated resistance arteries were cannulated and secured with two 17 μm nylon sutures to size-matched micro-cannulae, one of which is controlled by a micrometer and connected to the in-line force transducer. Following mounting, the vessel lumen was flushed gently with PSS to remove any blood or debris. The PSS was then gradually warmed to 37 °C. Over a period of 30 min, arteries were then gradually pressurised from 10–40 mmHg. During all experiments the PSS was gassed with 95% O_2/5% CO_2 maintaining pH 7.4. Having obtained an intraluminal pressure of 40 mmHg, arteries were given a further period of 30 minutes in a no-flow state. At this stage, artery length was equilibrated longitudinally. Utilising the longitudinal force transduction measurement the micrometer was used to equilibrate vessel length to a point of "no slack/no force" (0 mN). Arteries were then activated with 60 mM potassium-containing PSS (high potassium PSS) three times, then washed with PSS and allowed a further 15 min. Pharmacological studies were constructed at an intraluminal pressure of 40 mmHg in a no-flow state. Non-cumulative concentration responses were measured to a range of KPSS concentrations (4.5 mM, 10–100 mM in increments of 10 mM). Potassium chloride (KCl) concentration was

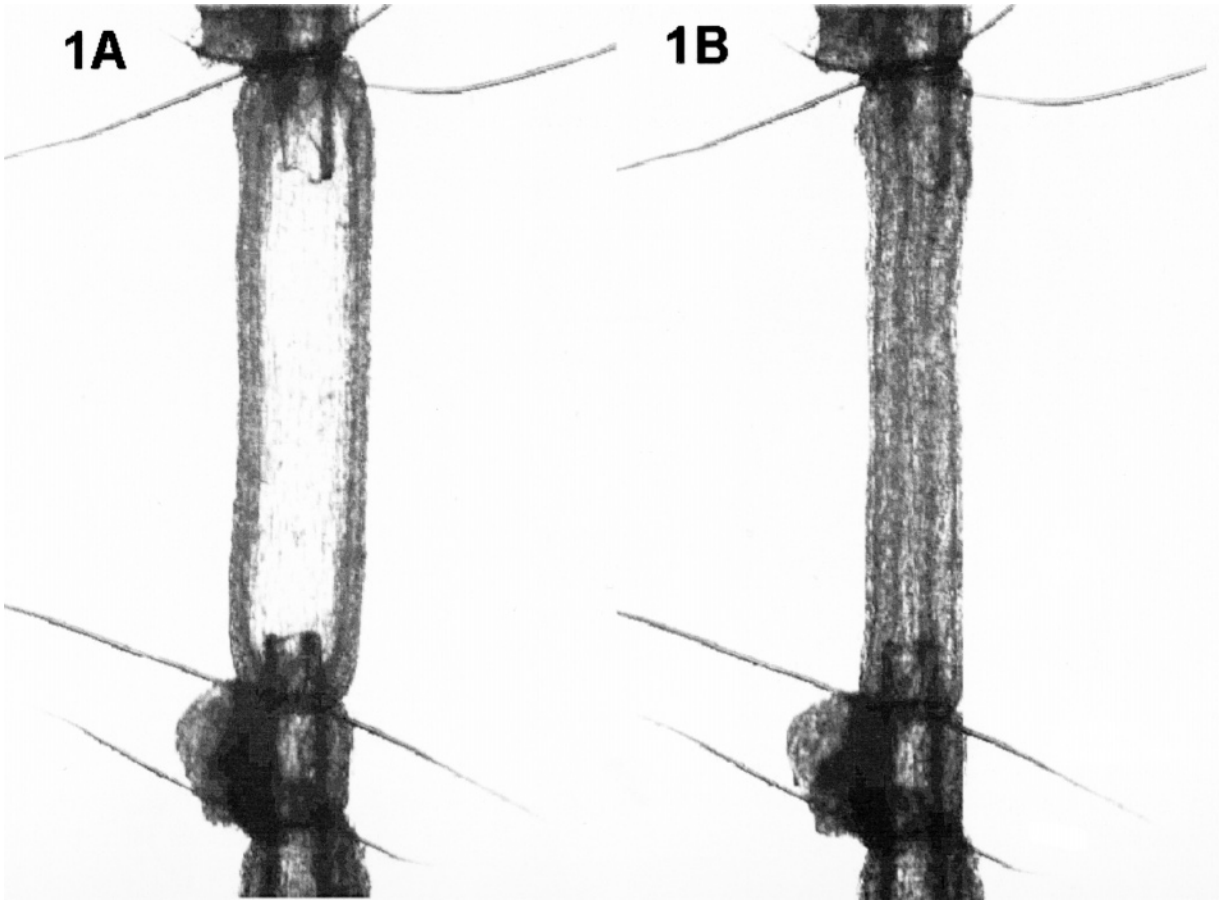

Fig. 1. (A) This shows an isolated resistance artery cannulated on a pressure myograph system in a passive state. (B) This shows the same artery in its maximum active state.

adjusted in the PSS from 4.5 mM–100 mM by equimolar substitution of sodium chloride for KCl. Vasoconstrictor responses were also constructed to noradrenaline (NA) and phenylephrine (PE). These were studied, since noradrenaline is the most likely endogenous vasoconstrictor *in vivo* and the α_1-adrenoceptor-specific agonist phenylephrine would allow the isolation of this component of the response. Effect on NA re-uptake mechanisms was studied by repeating the NA curves following a 30-min incubation period with 3 μM cocaine.[12,13] Data are represented as percentage contraction relative to the maximal KPSS contraction. Contraction data to non-cumulative addition of KPSS are represented as percent reduction in diameter relative to the maximum KPSS response.

Drugs and solutions

All drugs and solutions were prepared fresh on the day of the experiment. All were dissolved in distilled water and purchased from Sigma (Poole, Dorset, UK). Physiological saline solution (PSS) had the following composition in mM–NaCl 119, KCl 4.5, $NaHCO_3$ 25, KH_2PO_4 1.0, $MgSO_47H_2O$ 1.0, Glucose 6.0 and CaCl 2.5. High-potassium PSS (KPSS) composition: equimolar substitution of NaCl with KCl, otherwise identical to PSS.

Data and statistical analysis

Data was imported directly to Microsoft Excel 97 for IBM. Statistical analysis of the data was performed using Microsoft Excel 97 and Prism software (GraphPad, San Diego, CA, U.S.A.). Statistical analyses of the concentration required to produce a 50% response (EC_{50}) and maximal response between proximal and distal arteries were performed using the Student's paired *t*-test. Concentration response curves were analysed by one-way analysis of the variance (ANOVA) for repeated measures. Significance was assumed if $p<0.05$. Values are expressed as mean values $\pm$ S.E.M.

Table 1. Morphological characteristics of arteries isolated from amputated human legs. CSA: cross-sectional area. *$p<0.05$, proximal vs. distal.

	PS ($n=14$)	DS ($n=14$)	PSM ($n=12$)	DSM ($n=12$)
Lumen diameter (μM)	166.7±8	175.9±9	174.2±10	178.3±15
Wall thickness (μM)	29.7±2	21.3±1*	35.1±2	28.7±3*
Wall CSA (μM^2)	17966±1194	13349±1360*	23135±1126	19264±1765*
Wall:lumen (%)	18.6±2	12.3±1*	20.6±1	17.0±2

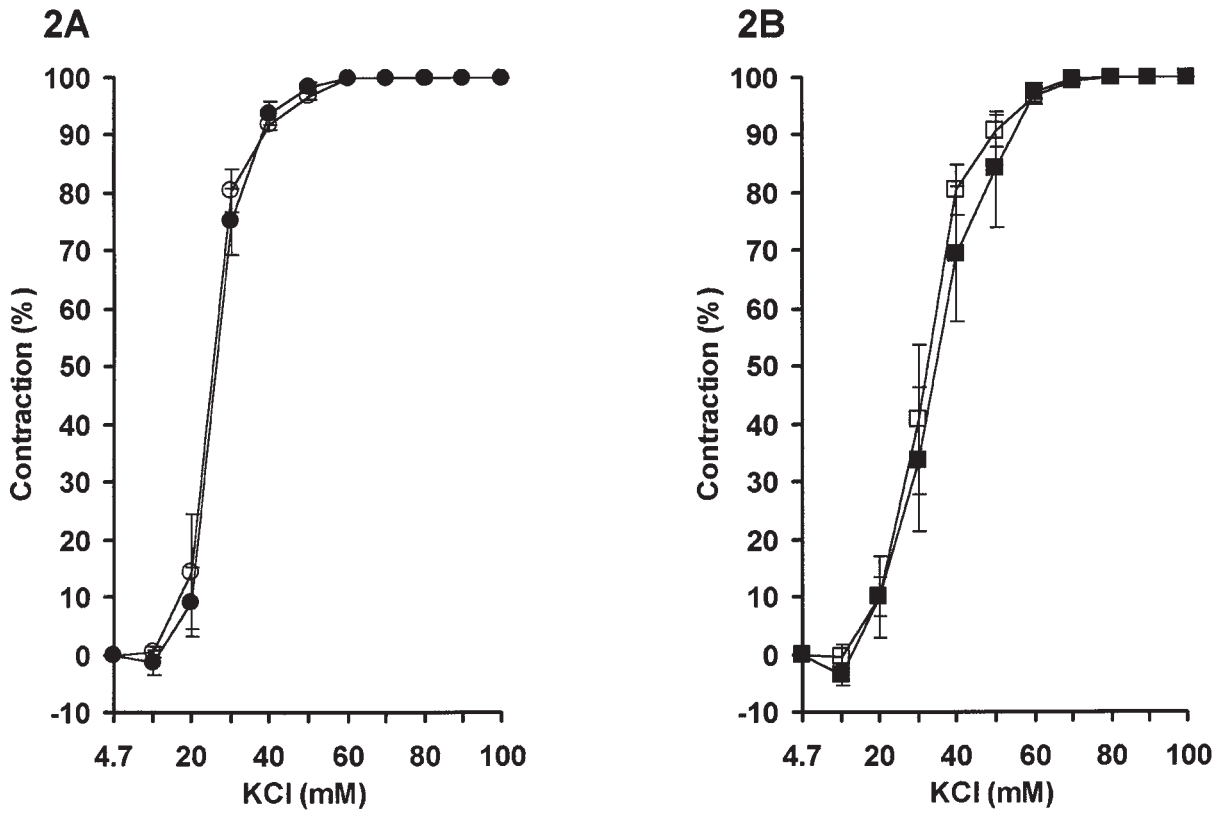

Fig. 2. (A) Response to non-cumulative addition of KCl-adjusted PSS in PS (●) vs. DS (○) arteries, $n=10$ pairs. (B) PSM (■) vs. DSM (□) arteries, $n=12$ pairs.

Results

Table 1 shows the morphological characteristics of the arteries from proximal subcutaneous (PS), distal subcutaneous (DS), proximal skeletal muscle (PSM) and distal skeletal muscle (DSM) sites. The mean lumen diameters were similar in proximal and distal sites whether from subcutaneous or skeletal muscle beds. However, comparison of the cross-sectional area and wall thickness highlighted a significant reduction in the distal vessels from both sites ($p<0.05$). This accounted for a reduction in wall thickness of ~30% in the subcutaneous bed and ~20% in the skeletal muscle bed.

The response to non-cumulative addition of KPSS in PS arteries and DS arteries are shown in Figure 2A. EC_{50} values were PS: 26.2±0.9 mM and DS: 25.6±1.7 mM. Similarly, no significant difference was found between sites in the skeletal muscle bed (Fig. 2B) with C_{50} values of PSM: 35.2±3.8 mM and 31.1±2.7 mM. Therefore, there was no difference in the ability of vessels from either site to vasoconstrict in response to electromechanical stimuli.

Figure 3 shows the result of studies of cumulative addition of NA in the vessels. Comparison of PS responses versus DS responses (Fig. 3A) highlights a significantly attenuated vasoconstriction response to NA in the distal arteries. Both maximum vasoconstriction (PS: 111.7±5.4%; DS: 90.8±4.1%, $p<0.05$) and sensitivity (EC_{50}) of the vessels to NA were reduced (EC_{50} – PS: 0.087±0.02 μM; DS: 0.31±0.11 μM, $p<0.05$). Similar studies on skeletal muscle vessels produced

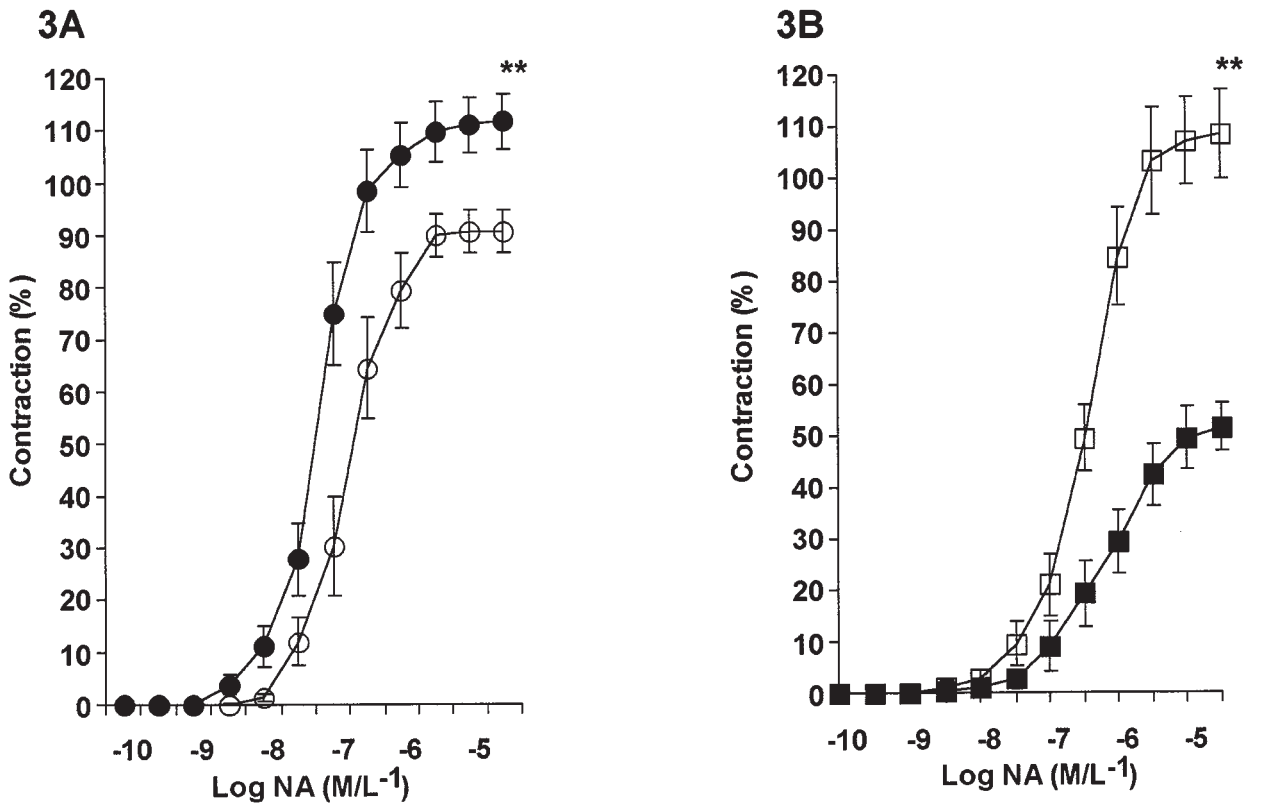

Fig. 3. (A) This shows the response to cumulative addition of NA in PS(●) vs. DS (○) arteries, $n=12$ pairs. (B) PSM (■) vs. DSM (□) arteries, $n=12$ pairs. **$p<0.01$.

strikingly different results (Fig. 3B). A clear and significant augmentation of NA vasoconstriction was apparent in the DSM. The maximal response to NA observed in PSM arteries was $51.5\pm4.6\%$ compared with $108.2\pm8.6\%$ in DSM arteries ($p<0.05$) while the EC_{50}s were PSM: $2.11\pm1.24\,\mu M$ and DSM: $0.36\pm0.05\,\mu M$ ($p<0.05$).

The studies using the α_1-selective adrenoceptor agonist phenylephrine gave similar results to those obtained from the NA studies. In response to cumulative addition of PE there was again a significant loss of response observed in DS arteries compared with PS arteries (Fig. 4A, $p<0.05$). Maximal vasoconstriction in the PS arteries was $93.0\pm1.8\%$ and only $79.5\pm3.2\%$ in DS arteries. Furthermore, the reduction in response resulted in a significant attenuation in the sensitivity of the vessels to PE, with EC_{50} values of $0.71\pm0.14\,\mu M$ in the PS and $1.45\pm0.26\,\mu M$ in the DS ($p<0.05$). Once again, in response to PE, skeletal muscle arteries produced a significant and opposite response to those observed in the subcutaneous arteries (Fig. 4B). The PSM produced a maximal response of $34.4\pm5.6\%$ as compared to $112.3\pm5.9\%$ in the DSM ($p<0.01$) with, in this case, a significant increase in sensitivity shown by EC_{50} values of $7.5\pm2.28\,\mu M$ in PSM and $0.48\pm0.09\,\mu M$ in DSM ($p<0.05$).

Table 2 summarises the results of separate sets of cumulative concentration response curves to NA followed by NA in the presence of 3 μM cocaine. Cocaine blockade of NA re-uptake was shown to increase the maximum responses of the tissue to NA in PS, DS and PSM vessels; however, no significant effect of cocaine was observed in DSM vessels. This indicates some impairment of NA re-uptake in these vessels. In PS arteries the maximum response increased from $100.2\pm4.1\%$ to $114.2\pm5.3\%$ ($p<0.05$), with a non-significant change in EC_{50} from $0.19\pm0.06\,\mu M$ to $0.26\pm0.04\,\mu M$. In the DS arteries the maximum response to NA was increased from $88.3\pm4.2\%$ to $100.8\pm2.3\%$ post-incubation ($p<0.05$) with no change in the EC_{50} values. Similarly, in PSM arteries cocaine increased the maximum response to NA from $43.9\pm5.8\%$ to $54.8\pm4.3\%$ ($p<0.05$). However, in the DSM arteries the maximum response did not change significantly (from $111.4\pm9.2\%$ to $114.3\pm9.4\%$) and the EC_{50} values remained constant at 0.36 ± 0.12 pre-cocaine to 0.47 ± 0.18 post-cocaine.

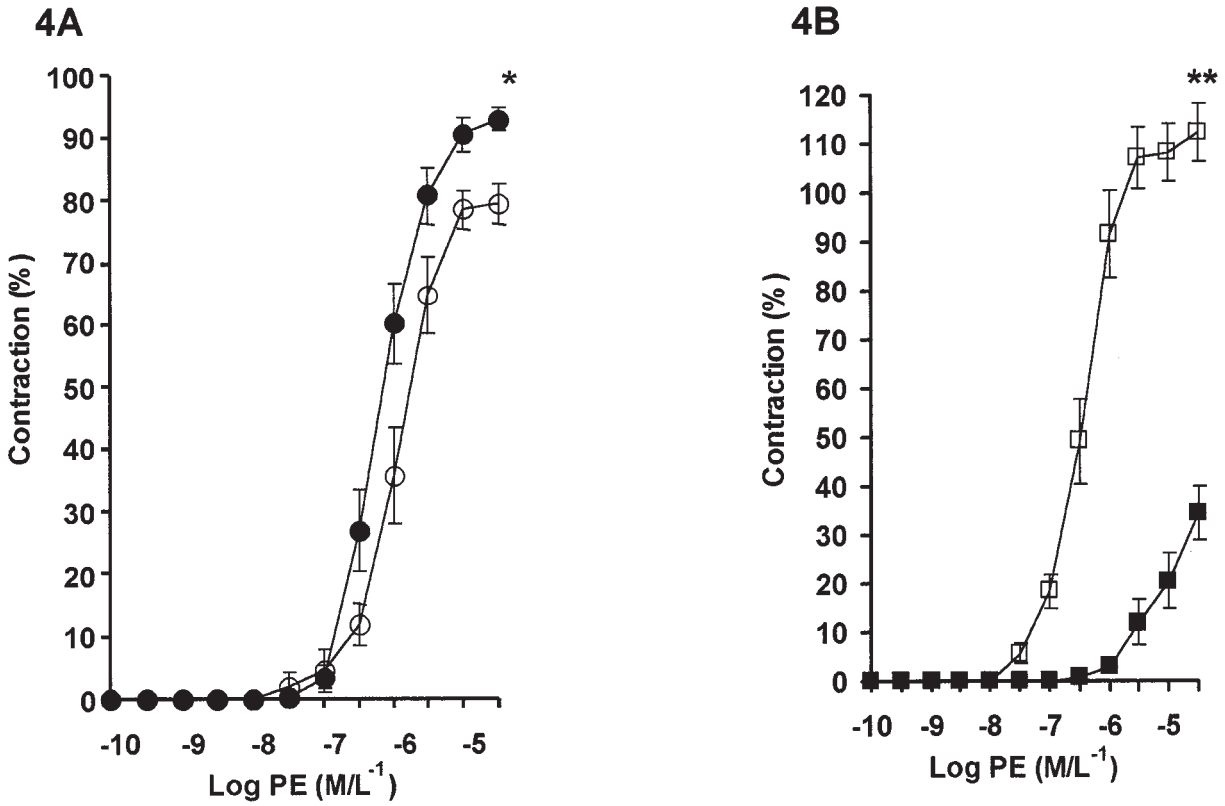

Fig. 4. (A) Response to cumulative addition of PE in PS (●) vs. DS (○) arteries, $n=10$ pairs. (B) PSM (■) vs. DSM (□) arteries, $n=10$ pairs. *$p<0.05$, **$p<0.01$.

Table 2. EC_{50} (μM) and maximum contraction (%) data in response to noradrenaline before and after incubation with 3 μM cocaine. PS vs. PS cocaine $n=6$ pairs, DS vs. DS cocaine $n=6$ pairs, PSM vs. PSM cocaine $n=6$ pairs and DSM vs. DSM cocaine $n=6$ pairs.

	EC_{50}	EC_{50} (cocaine)	p	Max	Max (cocaine)	p
PS	0.19±0.06	0.26±0.04	NS	100.2±4.1	114.2±5.3	<0.05
DS	0.49±0.29	0.68±0.52	NS	88.3±4.2	108.0±2.3	<0.05
PSM	2.16±0.82	1.84±0.94	NS	43.9±5.8	54.8±4.3	<0.05
DSM	0.36±0.12	0.47±0.18	NS	111.4±9.2	114.3±9.4	NS

Discussion

This study has identified significant structural differences between arteries derived from non-ischaemic sites and those derived from ischaemic sites on the affected limb of patients with critical limb ischaemia. These changes are observed as a general atrophy of the arterial wall. As well as structural effects, we have also identified functional differences between the two sites although, intriguingly, the abnormalities are differential depending on the vascular bed studied. Specifically, vasoconstrictor responses to adrenoceptor agonists are either attenuated, in vessels from the subcutaneous bed, or dramatically augmented as observed in the skeletal muscle bed. Studies looking at mechanism underlying these observations suggest that in both α_1-mediated vasoconstriction is affected and that in ischaemic skeletal muscle arteries the noradrenaline re-uptake mechanism may be impaired.

We have identified a significant arterial wall atrophy in vessels from ischaemic sites in subcutaneous and skeletal muscle vascular beds, confirming observation made previously in subcutaneous resistance arteries studied on the wire myograph.[10] This study has now revealed that this phenomenon is also apparent in the skeletal muscle vascular bed. A reduction in cross-sectional area around a similar-sized lumen in distal arteries compared to proximal arteries is indicative of wall atrophy and thus rules out remodelling of the arterial wall. Whether this observed phenomenon is due to smaller cells, less cells or a reduction in extracellular mass has not been determined as yet and

therefore merits a focused study. The structure of resistance arteries is highly correlated with their function as the main modulators of peripheral vascular resistance.[14] These small arteries of less than 200 μm diameter are the most relatively muscular of all blood vessels. Therefore, this observation is important, since it throws doubt upon the ability of atrophied vessels in an ischaemic bed to perform their role in reducing perfusion pressure before it reaches the fragile capillary beds. Although this is not a significant disability in the low pressure ischaemic environment, it may, however, be important on the return to systemic perfusion pressures that occur following revascularisation.

Physiologically, mechanisms which produce vascular wall atrophy in ischaemic conditions are logical, since it is generally believed that the controlling stimulus for maintaining structural integrity is intramural pressure.[15] It is these mechanisms which, when faced with hypertension, result in over-stimulus and vessel wall growth or remodelling.[16] Hence, it may be that what we have observed in ischaemia is simply a part of the continuum of the system for maintaining a flexible arterial wall structure. Although complex, the transduction mechanisms involved almost certainly involve the switching on and off of a number of growth factors of which platelet-derived growth factor[17] and insulin-like growth factor[18] are considered important. Also, recent work has highlighted the role of the family of molecules involved in the homeostasis of extracellular matrix, including the matrix metalloproteinases and the tissue inhibitor metalloproteinases.[19,20] Ongoing work in vascular research should elucidate the importance of these factors in the process as well as those involved in cell migration and apoptosis.

This study uses vasoconstriction to KPSS to test the vasoconstrictor response to non-agonist-inducement. These results showed no difference in the sensitivity of either vascular bed to KPSS depolarisation. This is useful, since the subsequent finding of abnormalities of the more complex agonist-induced vasoconstriction response to NA and PE cannot be related simply to structural differences. Indeed, re-analysis of vasoconstriction responses to take into account the thinner walls obviously leads to even more strikingly increased vasoconstriction in skeletal muscle arteries.

It is difficult to be clear about the clinical importance of differential responses to NA and PE between subcutaneous arteries and skeletal muscle arteries. These data suggest that, in CLI patients, any situation which leads to an increase in plasma catecholamine levels would lead to a specific vasoconstriction in the skeletal muscle beds. This, coupled with an impaired response in subcutaneous vessels, would lead to blood being diverted from muscle to skin and may be the basis of a hypothesised redistribution phenomenon. A stress-induced rise in plasma catecholamines has been shown previously during a number of surgical procedures, including revascularisation surgery.[21] It is possible that a catecholamine-induced vasoconstriction of skeletal muscle is partly responsible for the no-reflow phenomenon observed postoperatively.[22] Alternatively, as has been proposed previously, the impaired responses in subcutaneous vessels coupled with the impaired vasoconstriction response may account for post-operative oedema formation.[10]

In order to fully understand the mechanisms at work in the ischaemic limb, it is important to take into account the differential role of the sympathetic nervous system in both vascular beds. Sympathetic stimulation, in health, produces a differential response between subcutaneous and skin arteries (vasoconstriction) and skeletal muscle arteries (vasodilation) to enable the classic "flight-or-fight" mechanism. Therefore, the reduced vasoconstrictor responses observed in proximal skeletal muscle (PSM) when compared with proximal subcutaneous (PS) vessels is what would be expected. The interesting observation, in terms of CLI, is that the skeletal muscle arteries vasoconstrict more powerfully to catecholamine activation.

The mechanisms behind these observations were investigated by analysing the α_1-adrenoceptor selectivity to the phenomenon and the integrity of the endogenous cocaine-sensitive noradrenaline re-uptake system. The parallel results observed with PE indicate that the changes associated with ischaemia in both beds clearly involve the α_1-subtype, although a component of the response may be occurring with other subtypes. If, indeed, this phenomenon is clinically important, this suggests some therapeutic role for α_1-selective inhibitors in CLI. The importance of impaired re-uptake in DSM arteries is difficult to establish. The change in tissue sensitivity following blockade of this mechanism with cocaine is small, $\sim$3% in this study, and may be more involved with sustaining a contraction *in vivo* rather than increasing maximum response. Also, it is possible that the reduction in re-uptake observed in the DSM is a normal response to modulate the increased vasoconstriction produced by a change in tissue sensitivity. Therefore, if an increased vasoconstriction in distal skeletal muscle is considered detrimental to ischaemic perfusion, then an attenuated re-uptake phenomenon in that tissue may be a useful mechanism.

This study has reported a number of responses

which are hypothesised to be the result of chronic ischaemia. It is clear that the sites of biopsy for DSM and DS arteries are from an environment of chronic ischaemia. However, in order to be sure of cause and effect, it is necessary to compare ischaemic tissue with control non-ischaemic tissue. Previous studies have compared the structure and function of ischaemic subcutaneous arteries with normal non-ischaemic control tissue from patients with no ischaemia.[10] These have shown that the structure and function of PS and DS arteries in health are extremely similar. In that tissue, there is no difference between vascular beds in response to vasoconstriction with noradrenaline and other agonists. In this study, it was not possible to obtain external controls of skeletal muscle biopsies from healthy people. Therefore, we have used vessels from the proximal sites as internal controls. However, in the case of the skeletal muscle data, in the absence of external controls, even though it is not possible to be absolutely sure that the observations are the result of ischaemia, the observations themselves are still important, for the reasons outlined above.

In this study, we classified vessels derived from the incision level of both above- and below-knee amputations as non-ischaemic "proximal". Data from these arteries were pooled together, since previous studies had observed no intrinsic differences in structure or function between arteries derived from thigh and calf sites.[10] Furthermore, subsequent *post hoc* comparison of our data (not shown) has confirmed no differences between these sites. Similarly, arteries from both diabetic and non-diabetic patients were studied and pooled. There is no previous evidence for structural changes in resistance-sized arteries in diabetics, although functional abnormalities have been previously described.[23] These provide evidence of alterations in endothelium-dependent vasorelaxation mechanisms. The comparisons made in the present study do not specifically address endothelial function. However, it is acknowledged that some modulation of the vasoconstriction response to noradrenaline is mediated via the endothelium, although this is considered to be small. Again, *post hoc* analysis of our data (not shown) has confirmed no differences between the observation in diabetic and non-diabetic-derived arteries.

This study has highlighted differences in structure and function between proximal and distal resistance arteries in the ischaemic limb. Also, the functional changes are different depending on the vascular bed studied. We believe these data are important and may explain both the poor perfusion observed postoperatively and contribute to the redistribution of available blood in the ischaemic limb.

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